PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	**FOR FURTHER ACTION** see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
L/VZ70/cm/3		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/EP 99/ 07405	24/09/1999	29/09/1998
Applicant		
LEUVEN RESEARCH & DEVELOPEMENT VZW et al.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 3 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

   a. With regard to the **language**, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

   [ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

   b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

   [ ] contained in the international application in written form.

   [ ] filed together with the international application in computer readable form.

   [ ] furnished subsequently to this Authority in written form.

   [ ] furnished subsequently to this Authority in computer readble form.

   [ ] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

   [ ] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [ ] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title**,

   [ ] the text is approved as submitted by the applicant.

   [X] the text has been established by this Authority to read as follows:

   USE OF COMPOUNDS THAT REDUCE ALPHA2-ANTIPLASMIN IN VIVO FOR THE PREPARATION OF A COMPOSITION FOR THE TREATMENT OF ISCHEMIC STROKE

5. With regard to the **abstract**,

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No.

   [ ] as suggested by the applicant.

   [ ] because the applicant failed to suggest a figure.

   [ ] because this figure better characterizes the invention.

   [X] None of the figures.

PATENT COOPERATION TREATY

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INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
L/VZ70/cm/3		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/EP99/07405	24/09/1999	29/09/1998
International Patent Classification (IPC) or national classification and IPC A61K39/395		
Applicant LEUVEN RESEARCH & DEVELOPEMENT VZW et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 7 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 1 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☒ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☒ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
27/04/2000	19.01.2001
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx. 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Maucher, C Telephone No. +49 89 2399 7415

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/EP99/07405

I. Basis of the report

1. This report has been drawn on the basis of *(substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments (Rules 70.16 and 70.17).)*:
Description, pages:

1-17	as originally filed				

Claims, No.:

2-6	as originally filed				
1	as received on	27/11/2000	with letter of	24/11/2000	

Drawings, sheets:

1/3-3/3	as originally filed				

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

   These elements were available or furnished to this Authority in the following language: , which is:

   - ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
   - ☐ the language of publication of the international application (under Rule 48.3(b)).
   - ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

   - ☐ contained in the international application in written form.
   - ☐ filed together with the international application in computer readable form.
   - ☐ furnished subsequently to this Authority in written form.
   - ☐ furnished subsequently to this Authority in computer readable form.
   - ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
   - ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

☐ the description, pages:

☐ the claims, Nos.:

☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

(Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

II. Priority

1. ☐ This report has been established as if no priority had been claimed due to the failure to furnish within the prescribed time limit the requested:

☐ copy of the earlier application whose priority has been claimed.

☐ translation of the earlier application whose priority has been claimed.

2. ☒ This report has been established as if no priority had been claimed due to the fact that the priority claim has been found invalid.

Thus for the purposes of this report, the international filing date indicated above is considered to be the relevant date.

3. Additional observations, if necessary:
see separate sheet

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	
	No:	Claims	1-6
Inventive step (IS)	Yes:	Claims	
	No:	Claims	1-6
Industrial applicability (IA)	Yes:	Claims	
	No:	Claims	

2. Citations and explanations
see separate sheet

VII. Certain defects in the international application

The following defects in the form or contents of the international application have been noted:
see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:
see separate sheet

The arguments filed by the applicant with a letter of 24.11.2000 have been taken into account for establishing this report.

Point II:

The subject-matter of the interfering document "CIRCULATION, vol. 99, no. 18, 11 May 1999 (1999-05-11), pages 2440-2444" does not refer to relevant subject-matter for novelty and inventive step (Articles 33(2) and (3) PCT) for the following reasons.

The above document is published after the present application's earliest priority date (29.9.98), but before its filing date and is therefore relevant for those parts of the present application, which do not have a valid claim to said priority, i.e. claim 5 concerning the feature "ScFv fragments" and complete claim 6.
However, neither the feature "ScFv fragments", nor the subject-matter of claim 6 is disclosed in said document.

Point V:

Reference is made to the following documents:

D1: WO-A-98 12329
D2: EP-A-0 631 786

1. Articles 33(2) and (3) PCT

1.1. D1 and D2 disclose treatments for infarction (D1: abstract: myocardial infarction, D2: claim 17) or stroke (D1: abstract; D2: page 2, lines 1-9) using α_2-antiplasmin binding compounds (D1: abstract; D2: page 4, line 46) for thrombolytic therapy which enhances fibrinolysis (D1, p 6, l 2-3) and prevention of ischemic damage of neural cells (D2, p 6, l 33-34).
Neither document discloses explicitly the wording "reduction of the size of the focal cerebral ischemic infarct".

However, the subject-matter of **independent claim 1** is not **novel** (Article 33(2) PCT) for the following reasons:
Infarction is a region of dead cells due to ischemia through thrombosis for instance. Such dead cells are neuronal cells if said infarction occurs in the brain. This fact is well-known in the art (see also D2, p 3, l 15-16, l 35-38, l 47-48). It is clear that the skilled person wants to combat the expansion of dead cells when treating infarction by thrombolysis for instance (see for instance D2, p 6, l 34; p 7, example 1). It is believed that the combat of expansion of the infarction (see also VIII, 3.) is the general goal of infarct treatments. Thus, the treatment methods of D1 and D2 are detrimental of novelty for present claim 1.

The same applies to dependent claims 2-6, which only disclose additional features well-known in the art:

- D1 discloses that specific antibodies to α_2-antiplasmin inhibit α_2-antiplasmin activity (page 5, lines 16-18). It appears that, due to the binding of said compounds, the concentration of (unbound) α_2-antiplasmin is also reduced. Therefore, **claims 2-3** are **not novel** in the light of D1.

- D1 and D2 disclose α_2-antiplasmin binding compounds like monoclonal antibodies, Fab fragments or scFv fragments of anti-α_2-antiplasmin antibodies (D1: page 24, lines 14-18) or plasmin (D2: abstract) for use in the treatment of infarction or stroke.
Therefore, **claims 4-6** are **not novel** in the light of D1 or D2.

2. Industrial Applicability

For the assessment of the present **claims 1-6**, which are directed to the use of a compound in the preparation of a medicament, on the question whether they are industrially applicable, no unified criteria exist in the PCT contracting States. The patentability can also be dependent upon the formulation of the claims. The EPO, for example, does not recognize as industrially applicable the subject-matter of claims to the use of a compound in medical treatment, but may allow, however, claims to a known compound for first use in medical treatment and the use of such a compound for

the manufacture of a medicament for a new medical treatment.

Point VII:

Contrary to the requirements of Rule 5.1(a)(ii) PCT, the documents **D1-D2** have not been identified in the description and the relevant background art disclosed therein have not been briefly discussed.

Point VIII:

1. The subject-matter of claim 1 does not meet the requirements of Article 6 PCT because it is unclear whether the expression "compounds that reduce α_2-antiplasmin" refers to "α_2-antiplasmin concentration" (see claim 2) or "activity" (see claim 3) or another parameter. This objection has not been overcome.

2. Furthermore, the subject-matter of claim 1 does not meet the requirements of Article 6 PCT, since it contains the feature "ischemic stroke" mentioned in brackets. Such features are considered to be purely optional. It appears from the abstract of D1 and from D2, p 3, l 15-16, that "infarction" and "stroke" are different diseases and that the feature in brackets is therefore not just an explanation of a term mentioned in the claim. It is thus not clear to which subject-matter the claim refers.

3. Moreover, claim 1 is not clear for the following reasons. According to page 1, a focal cerebral ischemic infarction leads to neuronal cell death. In view of said statement it is not clear how the size of an infarction - characterized by dead neuronal cells - can be reduced, i.e. how dead cells can be resuscitated.

Application No PCT/EP99/07405

Enclosure to letter dated 24 November 2000

NEW CLAIM 1

1. Use of compounds that reduce α_2-antiplasmin in vivo, for the preparation of a therapeutical composition for the treatment of focal cerebral ischemic infarction (ischemic stroke) by reducing the size of the focal cerebral ischemic infarct.

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International Bureau

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Published

With international search report.

Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: USE OF COMPOUNDS THAT REDUCE ALPHA2–ANTIPLASMIN IN VIVO FOR THE PREPARATION OF A COMPOSITION FOR THE TREATMENT OF ISCHEMIC STROKE

(57) Abstract

The present invention relates to a new means for the treatment of focal ischemic cerebral infarction (ischemic stroke). It has been found that reduction of α_2–antiplasmin leads to a significantly smaller focal cerebral infarct size. The invention therefore provides the use of compounds that reduce α_2–antiplasmin concentration or activity *in vivo*, for the preparation of a therapeutical composition for the treatment of focal cerebral ischemic infarction (ischemic stroke).

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USE OF COMPOUNDS THAT REDUCE ALPHA2-ANTIPLASMIN IN VIVO FOR THE PREPARATION OF A COMPOSITION FOR THE TREATMENT OF ISCHEMIC STROKE

The present invention relates to a new means for the treatment of focal ischemic cerebral infarction (ischemic stroke).

Focal ischemic cerebral infarction occurs when the arterial blood flow to a specific region of the brain is reduced below a critical level resulting in neuronal cell death. It is thought that neuronal degeneration in central nervous system (CNS) diseases such as stroke, epilepsy and Alzheimer's disease is stimulated by an excess of the excitatory amino acid glutamate (2). Injection of glutamate agonists in the CNS indeed induces hippocampal neuronal cell death similar to that observed in neurodegenerative diseases (3).

Excitotoxin-induced neuronal degeneration is mediated by tissue-type plasminogen activator (t-PA) (4). Consistent with this observation, mice deficient in t-PA are resistant to, and infusion of plasminogen activator inhibitor-1 (PAI-1) protects against excitotoxin-mediated hippocampal neuronal degeneration (4-6).

Furthermore, deficiency of plasminogen (Plg), the zymogen substrate of t-PA, and infusion of α_2-antiplasmin (α_2-AP), protect mice against excitotoxin-induced hippocampal neuronal death (5). It has been proposed that plasmin-mediated degradation of laminin sensitizes hippocampal neurons to cell death by disrupting neuron-extracellular matrix interaction (7).

Wang et al. (8) recently demonstrated that neuronal damage after focal cerebral ischemia induced by transient occlusion of the middle cerebral artery was also reduced in mice with t-PA deficiency and exacerbated by t-PA infusion. This suggests that the plasminogen system may be involved both in establishing a cerebral ischemic infarct and in its extension during thrombolytic therapy. It was recently demonstrated that the neurotoxic effect of t-PA on persistent focal cerebral ischemia also occurred with other thrombolytic agents, including

streptokinase and staphylokinase (9). Thus, in those patients with persistent cerebral arterial occlusion, thrombolytic therapy for ischemic stroke may cause infarct extension, which would not only partially offset the established overall beneficial effect of arterial recanalization (10, 11), but indeed be harmful to a subgroup of patients. Because it is not possible to distinguish between patients who will and those who will not achieve cerebral arterial recanalization with thrombolytic therapy, the development of specific conjunctive strategies to counteract the neurotoxic effects of thrombolytic agents on persisting focal cerebral ischemia appear to be warranted.

It is therefore the object of the present invention to provide a new means for treating ischemic stroke.

In the research that led to the present invention the following was contemplated. Although it is assumed that neuronal injury during focal ischemia in the brain occurs primarily as a result of accumulation of excitotoxins such as glutamates, the role of plasmin-mediated laminin degradation or alternative mechanisms in the pathogenesis of cortical neuronal cell death has not been demonstrated. In order to delineate the contribution of individual components of the plasminogen (fibrinolytic) system on focal cerebral ischemic infarction, the present inventors then quantitated infarct size produced by ligation of the left middle cerebral artery (MCA) in mice with targeted inactivation of the genes encoding Plg, its activators tissue-type plasminogen activator (t-PA) or urokinase-type plasminogen activator (u-PA), or the fibrinolytic inhibitors PAI-1 or α_2-AP. In addition, the effects of adenoviral transfer of the t-PA and PAI-1 genes and of infusion of human α_2-AP on cerebral infarction were studied.

Whereas the findings of Strickland et al., that t-PA deficiency protects against focal cerebral ischemic

infarction were fully confirmed, and extended by the observation that PAI-1 deficiency resulted in significantly larger infarct sizes, the observation that Plg deficiency protects against excitotoxin induced neuronal cell death could not be confirmed. Instead it was found that focal cerebral infarct size was significantly larger in mice with Plg deficiency and conversely, significantly smaller in mice with α_2-AP deficiency.

In aggregate, these findings indicate that plasminogen system components affect focal cerebral ischemic infarct size at two different levels: 1) reduction of t-PA activity (t-PA gene inactivation or PAI-1 gene transfer) reduces, while its augmentation (t-PA gene transfer or PAI-1 gene inactivation) increases infarct size, and 2) reduction of Plg activity (Plg gene inactivation or α_2-AP injection) increases, while its augmentation (α_2-AP gene inactivation or α_2-AP neutralization) reduces infarct size. The findings are incompatible with a unique linked pathway in which t-PA-mediated plasmin generation would lead to neuronal cell death, but suggests two independent (t-PA mediated and Plg-mediated, respectively) mechanisms operating in opposite direction.

The internally consistent observations with α_2-AP were unexpected but are most relevant for the treatment of ischemic stroke. Firstly a correlation was found between infarct size and genotype with heterozygotes displaying infarct sizes between those of the wild type and homozygous phenotypes. Secondly, bolus injection of human α_2-AP (hα_2-AP) in α_2-AP$^{-/-}$ mice caused a dose-related infarct expansion. Importantly, Fab fragments from affino-specific polyclonal rabbit anti-hα_2-AP antibodies, given intravenously 40 min after occlusion of the MCA, significantly reduced the cerebral ischemic infarct size. This observation suggests a potential avenue to counteract focal ischemic infarction with the use of α_2-AP inhibitors (e.g. neutralizing monoclonal antibodies or compounds neutralizing α_2-AP

activity). This approach was confirmed by infusion of plasmin in mice with MCA occlusion which, by neutralizing α_2-AP, significantly reduced infarct size. The concentration of α_2-AP in human plasma is 1 mM (12), corresponding to a total body pool of approximately 500 mg. An equivalent dose of a monoclonal Fab fragment would be approximately 400 mg, and that of plasmin approximately 500 mg, which is high but not excessive for single therapeutic administration. Furthermore, the observation that infarct size is proportional to the α_2-AP level (derived from the gene dose effect and the dose-response) suggests that a partial reduction of the plasma level might have a significant beneficial effect.

In view of the above the invention thus relates to the use of compounds that reduce α_2-AP activity in vivo for the treatment of focal cerebral ischemic infarction (ischemic stroke).

In a specific embodiment of the invention use is made of compounds that reduce the circulating α_2-AP concentration. A lower concentration of α_2-AP will lead to a lower activity. In an alternative embodiment, the activity of circulating α_2-AP is reduced directly.

Compounds that are suitable for the reduction of α_2-AP concentration and activity are for example α_2-AP neutralizing antibodies or derivatives thereof. Preferred antibodies are monoclonal antibodies. Derivatives are preferably Fab fragments, scFv fragments.

Compounds neutralizing α_2-AP are for example plasmin, mini-plasmin (lacking the first 4 kringles) or micro-plasmin (lacking all five kringles).

The present invention will be demonstrated in more detail in the following examples, which are however not intended to be limiting to the scope of the invention. In the examples reference is made to the following drawings:

Figures 1 to 3 are histograms comparing the volume (in mm^3) of focal cerebral ischemic infarcts after ligation of the middle cerebral artery (MCA) in mice. The

data represent mean values and the vertical bars SEM, with the number of experiments given in the columns.

Figure 1 shows the effect of deficiency of plasminogen system components (genotype in abscissa) on focal ischemic cerebral infarct size (in mm^3). WT: wild type (pooled values of 50% C57BL6/50% S129, 100% C57BL6 and 100% S129 genetic background).

Figure 2 shows the effect of adenoviral transfer of the t-PA or PAI-1 genes on focal ischemic cerebral infarct size in t-PA or PAI-1 deficient mice, respectively.

Figure 3 shows the effect of α_2-AP on focal ischemic cerebral infarct size.

A. Effect of α_2-AP genotype on cerebral infarct size.

B. Effect of injection of hα_2-AP or of h$\alpha\alpha_2$-AP followed by anti-hα_2-AP Fab fragments on cerebral infarct size.

EXAMPLES

EXAMPLE 1

Murine cerebral ischemic infarction model

1. Introduction

All mice included in the present study were generated and bred at the Specific Pathogen Free Facility of the Center for Transgene Technology and Gene Therapy, Campus Gasthuisberg, K.U. Leuven. Gene inactivation was obtained by homologous recombination in embryonic stem cells targeting the genes encoding tissue-type plasminogen activator (t-PA) (13), urokinase-type plasminogen activator (u-PA) (13), plasminogen activator inhibitor-1 (PAI-1) (14, 15), plasminogen (Plg) (16), or α_2-antiplasmin (α_2-AP) (17), as previously described. Mice with inactivated genes encoding u-PA receptor (u-PAR) (18) were not included because of the normal results obtained with u-PA deficient mice.

2. Materials and methods

2.1 Materials

Human α_2-AP was prepared from fresh frozen plasma as previously described (19).

Polyclonal antisera were raised in rabbits by subcutaneous injection of 200 mg purified human α_2-AP suspended in complete Freund's adjuvant, followed at two biweekly intervals by injection of the antigen suspended in incomplete Freund's adjuvant. Serum was obtained by repeated ear vein puncture. Pooled sera were chromatographed on Protein-A Sepharose (0.5 ml serum per ml wet gel), equilibrated with 0.1 M Tris.HCl, pH 8.1 and IgG eluted with 0.1 M glycine.HCl, pH 2.8, yielding approximately 10 mg protein per ml serum. Affino-specific antibodies were obtained from the dialyzed IgG pool by chromatography on a CNBr-activated Sepharose column substituted with human α_2-AP (2.5 mg/ml wet gel) and eluted with 0.1 M glycine.HCl, pH 2.8, yielding approximately 0.1 mg specific IgG per mg applied.

Fab fragments were obtained from the affino-specific IgG by digestion with papain. Therefore IgG was dissolved to a concentration of 5 mg/ml and digested with 1 percent (w/w) papain in the presence of 50 mM cysteine, 1 mM EDTA, 0.1 M phosphate buffer, pH 7.0 for 5 hours. The reaction was arrested by addition of iodoacetamide to a final concentration of 75 mM. After dialysis the mixture was purified on a protein A Sepharose column equilibrated with PBS. Fab concentration was determined by ELISA calibrated against an IgG standard. SDS gel electrophoresis essentially revealed homogeneous Fab fragments (not shown).

2.2 Production of adenoviral vectors

The recombinant adenoviruses AdCMVt-PA and AdCMVPAI-1 were generated by homologous recombination in 293 cells essentially as previously described (20). For AdCMVt-PA, an XbaI-fragment of the plasmid pSTEt-PA encoding wild type human t-PA was ligated into

XbaI-digested pACCMVpLpA (21) to produce pACCMVt-PA. The adenovirus precursor pACCMVPAI-1 was generated by ligating the 1.4-kb EcoRI/BglII fragment of pPAI-1RBR containing the entire coding sequence of human PAI-1 into EcoRI/BamHI-digested pACCMVpLpA. In these plasmids, the t-PA and PAI-1 cDNA are positioned between the human cytomegalovirus immediate-early enhancer/promoter and the SV40 t-antigen intron/polyadenylation signal to form a complete transcriptional unit.

Monolayer cultures of 293 cells (22) were cotransfected with 10 mg of pACCMVt-PA or pACCMVPAI-1 and 5 mg of pJM17 (20), a plasmid containing a full-length adenovirus 5 dl309 genome. Homologous recombination between these plasmids results in the formation of recombinant viral genomes in which the adenovirus E1 region is replaced by the respective t-PA or PAI-1 transgenes. Replication of the recombinant viruses in cultured 293 cells is supported by E1A gene products supplied in trans from a copy of E1 integrated into the 293 cell genome.

After transfection, recombinant viral plaques were harvested and amplified as described (23). The identity of recombinant viruses was determined by restriction analysis and Southern blotting of viral DNA prepared from productively infected 293 cells. The recombinant adenovirus AdRR5, which lacks an inserted gene in the E1 position, was generated from pACRR5 and pJM17 in the same manner and was used as a control adenovirus (24, 25). Recombinant viruses were replaqued to ensure clonal identity before further use. Large scale production of recombinant adenovirus was performed as described (23). Purified virus was supplemented with 0.1 mg/ml sterile bovine serum albumin (BSA), snap frozen in liquid nitrogen and stored at -80°C until use. The titer of infectious viral particles in purified stocks was determined by plaque assay on monolayers of 293 cells with 1 hour of adsorption at 37°C. Purified viral stocks of $>10^{10}$ plaque forming units (pfu) per ml were routinely

obtained. The kinetics and organ distribution of t-PA and PAI-1 expression following adenoviral transfer by intravenous bolus injection have been described elsewhere (26, 27).

2.3 Preparation of human plasmin

Human plasminogen was prepared from fresh frozen human blood bank plasma, essentially as described previously (28). Human plasma (6 liter), to which 20 units aprotinin (Trasylol, Bayer, Germany) was added per ml, was cleared by centrifugation at 4,000 rpm for 15 min at 4°C. Lysine-Sepharose (200g wet weight, substitution level approximately 1 mg lysine per g wet Sepharose gel) was added to the supernatant, the mixture stirred for 1 hour at 4°C and the gel recovered on a Buchner funnel. Then 120 g Lysine-Sepharose was added to the filtrate, the mixture stirred and the gel recovered as above. The combined gel fractions were washed with 18 liter 0.2 M K_2HPO_4/KH_2PO_4 buffer, pH 7.5, containing 10 units aprotinin per ml, then poured into a 5 x 60 cm column and washed with 0.02 M NaH_2PO_4, 0.1 M NaCl buffer, pH 7.5, containing 10 units/ml aprotinin at 4°C until the absorbance of the wash fluid at 280 nm was less than 0.05. The column was then eluted with wash buffer containing 0.05 M 6-aminohexanoic acid and protein containing fractions pooled. From 6 liter plasma approximately 145 ml fluid containing 650 mg protein was obtained. The pool was concentrated 2.5-fold on an Amicon PM10 filter and gel filtered on a 5 x 90 cm column of ultragel AcA44 equilibrated with 0.02 M NaH_2PO_4, 0.1 M NaCl buffer, pH 7.5, at a rate of 60 ml per hour. The main peak, containing approximately 590 mg protein was concentrated on an Amicon PM10 filter to a concentration of 10 mg/ml and frozen until use.

Human plasmin was prepared from plasminogen as follows. Lysine-Sepharose (20 g wet gel) was added to human plasminogen (200 mg) solution, the mixture stirred for 3 hours at 4°C, the gel washed on a Buchner funnel

and resuspended in 30 ml 0.1 M NaH_2PO_4 buffer, pH 7.4. Urokinase (500 µl of a 50 µM solution, prepared by activation of Saruplase (Grünenthal, Aachen, Germany) with Plasmin.Sepharose was added and the mixture stirred for 15 hours at 4°C. The gel was then washed on a Buchner funnel with 0.1 M NaH_2PO_4 buffer, pH 7.4, poured into a 1.5 x 16 cm column, washed with 0.1 M NaH_2PO_4 buffer, pH 7.4 until the absorbance at 280 nm of the wash fluid was less than 0.05, and eluted with 0.1 M NaH_2PO_4 buffer containing 0.05 M 6-aminohexanoic acid. The protein containing fractions were pooled, glycerol was added to a final concentration of 10 percent and the pool was dialyzed at 4°C against 0.1 M NaH_2PO_4 buffer containing 10 percent glycerol. The final recovery was 25 ml solution with a protein concentration of 4.0 mg/ml and an active plasmin concentration of 25 µM.

2.4 Measurement of α_2-antiplasmin in plasma

α_2-Antiplasmin levels in murine plasma were measured by a chromogenic substrate assay, based on its rapid inhibition of plasmin (29). Briefly 10 µl mouse plasma (diluted 1/10 in 0.05 M NaH_2PO_4 buffer, pH 7.4, containing 0.01% Tween 20) is mixed at 37°C with 420 µl 0.05 Tris HCl, 0.1 M NaCl buffer, pH 7.4, containing 0.01% Tween 20, and with 20 µl of 0.125 µM human plasmin (final concentration 5 nM). After 10s incubation, 50 µl of 3 mM S2403 (Chromogenics, Antwerp, Belgium) is added and the change in absorbance measured at 405 nm. The change in absorbance is approximately 0.18 min^{-1} with buffer and 0.09 min^{-1} with pooled murine plasma.

2.5 Animal experiments

Animal experiments were conducted according to the guiding principles of the American Physiological Society and the International Committee on Thrombosis and Haemostasis (30).

Focal cerebral ischemia was produced by persistent occlusion of the MCA according to Welsh et al. (31). Briefly, mice of either sex, weighing 20 to 30 g, were anesthetized by intraperitoneal injection of ketamine (75 mg/ml, Apharmo, Arnhem, The Netherlands) and xylazine (5 mg/ml, Bayer, Leverkusen, Germany). Atropine (1 mg/kg; Federa, Brussels, Belgium) was administered intramuscularly, and body temperature was maintained by keeping the animals on a heating pad. A "U" shape incision was made between the left ear and left eye. The top and backside segments of the temporal muscle were transsected and the skull was exposed by retraction of the temporal muscle. A small opening (1 to 2 mm diameter) was made in the region over the MCA with a hand-held drill, with saline superfusion to prevent heat injury. The meningae were removed with a forceps and the MCA was occluded by ligation with 10-0 nylon thread (Ethylon, Neuilly, France) and transsected distally to the ligation point. Finally, the temporal muscle and skin were sutured back in place.

AdCMVt-PA, AdCMVPAI-1 or AdRR5 were given as an intravenous bolus of 1.3×10^9 plaque forming units (p.f.u.) 4 days before ligation of the MCA. Human α_2-AP (hα_2-AP) was given intravenously divided in 2 injections, given 1 min before and 30 min after ligation of the MCA, respectively. Fab fragments were injected intravenously as a bolus, 10 min after the second hα_2-AP injection. Human plasmin was given intravenously as a bolus, either 15 min before or 15 min after ligation of the MCA.

The animals were allowed to recover and were then returned to their cages. After 24 hours, the animals were sacrificed with an overdose of Nembutal (500 mg/kg, Abbott Laboratories, North Chicago, IL) and decapitated. The brain was removed and placed in a matrix for sectioning in 1 mm segments. The sections were immersed in 2% 2,3,5-triphenyltetrazolium chloride (TTC) in saline (32), incubated for 30 min at 37°C, and placed in 4 % formalin in phosphate buffered saline. With this

procedure, the necrotic infarct area remains unstained (white) and is clearly distinguishable from stained (brick red) viable tissue. The sections were photographed and subjected to planimetry. The infarct volume was defined as the sum of the unstained areas of the sections multiplied with their thickness.

The data are represented as mean ± SEM of n determinations. The significance of differences was determined using analysis of variance followed by Fisher's PLSD test, using the Statview software package or by Student's t-test.

EXAMPLE 2

Cerebral ischemic infarct size in mice with targeted inactivation of genes encoding plasminogen system components

Ligation of the left MCA induced a cerebral infarct with a volume of 7.6 ± 1.1 mm^3 (n= 11) in wild type mice with a mixed (50%) S129 and (50%) C57BL/6 genetic background, of 9.3 ± 2.7 mm^3 (n= 6) in inbred C57BL/6 mice and of 6.4 ± 1.3 mm^3 (n= 6) in inbred S129 mice (p= NS versus mixed background, results not shown).

Inactivation of the t-PA gene was associated with a significant reduction of infarct size to 2.6 ± 0.80 mm^3 (n= 11), (p< 0.0001 vs wild type mice), whereas inactivation of the u-PA gene had no effect on infarct size (7.8 ± 1.0 mm^3, n= 8, p= NS vs wild type).

Inactivation of the PAI-1 gene was associated with a significant increase in infarct size (16 ± 0.52 mm^3, n= 6, p< 0.0001 vs wild type) (Figure 1). In mice with inactivated Plg genes, cerebral infarct size was significantly larger than in wild type mice (12 ± 1.2 mm^3, n=9, p=0.037 vs wild type), whereas, conversely, in α_2-AP gene deficient mice, infarct size was markedly reduced (2.2 ± 1.1 mm^3, n= 7, p= 0.0001 vs wild type) (Figure 1).

EXAMPLE 3

Effect of t-PA and PAI-1 gene transfer on cerebral infarct size

Injection of 1.3 x 10^9 p.f.u. of AdCMVt-PA in 6 t-$PA^{-/-}$ mice 4 days before MCA ligation was associated with a cerebral infarct size of 6.0 ± 1.3 mm^3, significantly larger than the infarcts in 5 t-$PA^{-/-}$ mice injected with the control virus AdRR5 (1.8 ± 0.63, p= 0.028) (Figure 2A). Conversely, injection of 1.3 x 10^9 p.f.u. of AdCMVP-AI-1 in 5 PAI-$1^{-/-}$ mice was associated with a cerebral infarct size of 10 ± 1.4 mm^3, significantly smaller than the infarcts in 5 PAI-$1^{-/-}$ mice injected with the control virus AdRR5 (13 ± 1.0 mm^3, p= 0.019) (Figure 2B).

EXAMPLE 4

Effect of α_2-antiplasmin on cerebral infarct size

Cerebral infarct size correlated with α_2-AP gene dose, corresponding to 11 ± 2.0, 4.9 ± 2.0 and 2.2 ± 1.1 mm^3 in wild type, heterozygous and homozygous deficient mice, respectively (Figure 3A). Injection of human α_2-AP in groups of 4 α_2-$AP^{-/-}$ mice increased the infarct size to 13 ± 2.5 mm^3 (n= 4) with a 1 mg total dose and to 11 ± 1.5 mm^3 (n= 6) with a 0.2 mg total dose. Injection of 1.7 mg affino-specific Fab against human α_2-AP in mice given 0.2 mg human α_2-AP reduced the cerebral infarct size to 5.1 ± 1.1 mm^3 (n= 7, p= 0.0040 vs 0.2 mg human α_2-AP) (Figure 3B).

The above examples show that reduction of α_2-AP activity (reduced α_2-AP gene expression or reduction of circulating α_2-AP with inhibitors) reduces focal cerebral ischemic infarct size, such as encountered during ischemic stroke.

EXAMPLE 5

Effect of plasmin on cerebral infarct size

Injection of 50, 100 or 150 μg human plasmin (Pli) in mice weighing approximately 30 g decreased the α_2-AP levels in blood samples taken after 30 s to 67, 40 and 31 percent of baseline, respectively (mean of 2 mice, with less than 15 percent variability). Injection of 200 μg Pli in 3 mice reduced the plasma α_2-AP levels to 59 ± 4.8, 67 ± 4.4 and 70 ± 2.5 percent after 2, 4 and 6 hours respectively.

Ligation of the left middle cerebral artery (MCA) induced a cerebral infarct with a volume of 27 ± 1.3 mm^3 (n= 10) in inbred Balb/c mice, and of 16 ± 1.3 mm^3 (n= 12) in inbred C57BL/6 mice.

Injection of 0.2 mg Pli in Balb/c mice reduced the infarct size to 22 ± 1.0 mm^3 (n= 9) (p= 0.006 vs saline). Similar decreases were observed when the Pli injection was given 15 min before or 15 min after ligation of the MCA (Table 1). In C57Bl/6 mice, injection of 0.2 mg Pli reduced the infarct size to 10 ± 1.2 mm^3 (n= 12) (p= 0.004 vs saline).

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CLAIMS

1. Use of compounds that reduce α_2-antiplasmin in vivo, for the preparation of a therapeutical composition for the treatment of focal cerebral ischemic infarction (ischemic stroke).

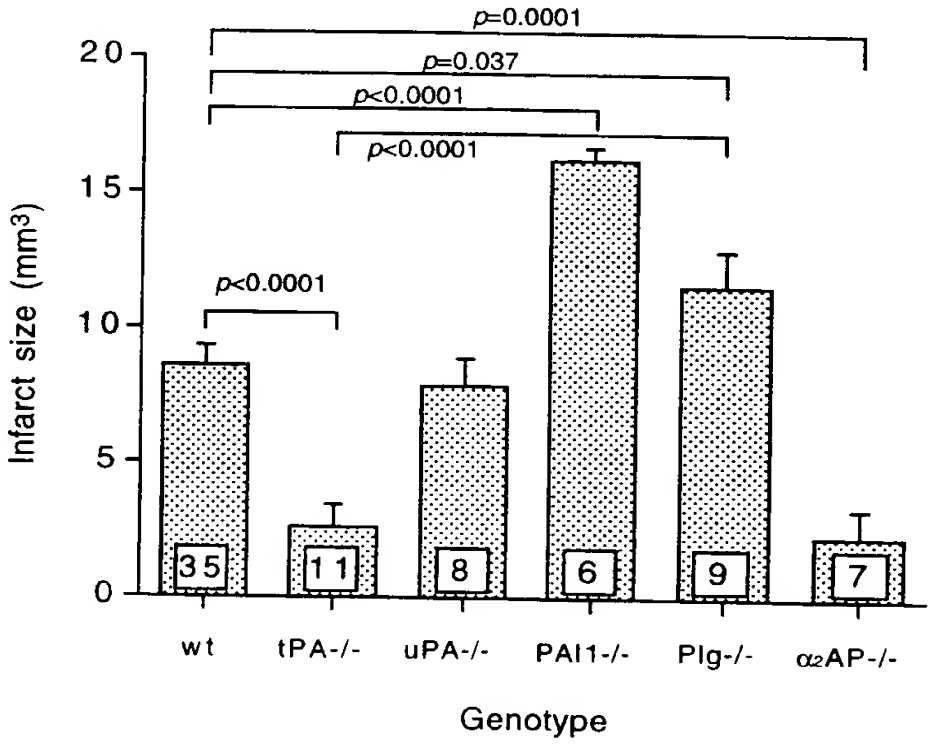
2. The use according to claim 1, wherein the compounds reduce the circulating α_2-antiplasmin concentration.

3. The use according to claim 1, the compounds reduce the circulating α_2-antiplasmin activity.

4. The use according to claims 1-3, wherein the compounds are α_2-antiplasmin neutralizing antibodies or derivatives thereof.

5. The use according to claim 4, wherein the derivatives are Fab fragments or ScFv fragments.

6. The use according to claims 1-3, wherein the compounds are α_2-antiplasmin neutralizing compounds selected from plasmin, mini-plasmin (lacking the first four kringles) or micro-plasmin (lacking all five kringles).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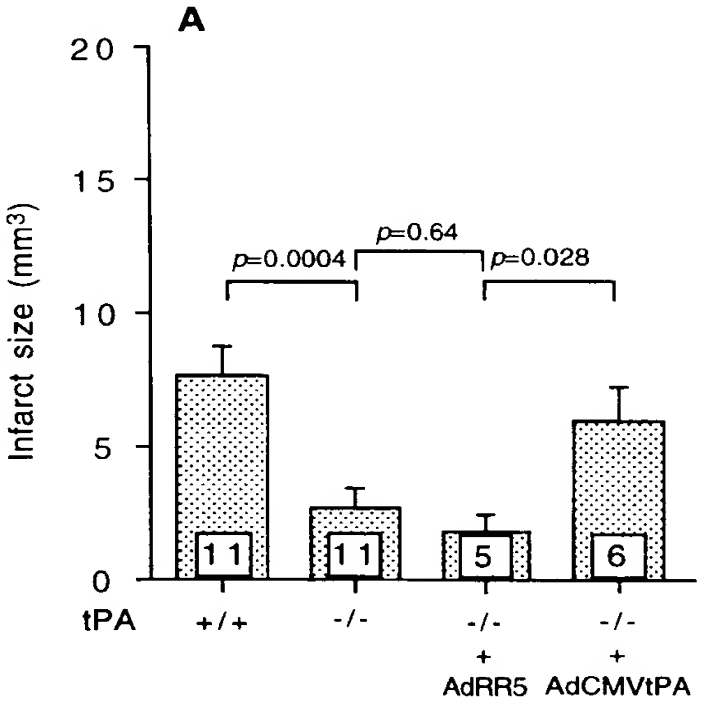
Genotype

FIG. 1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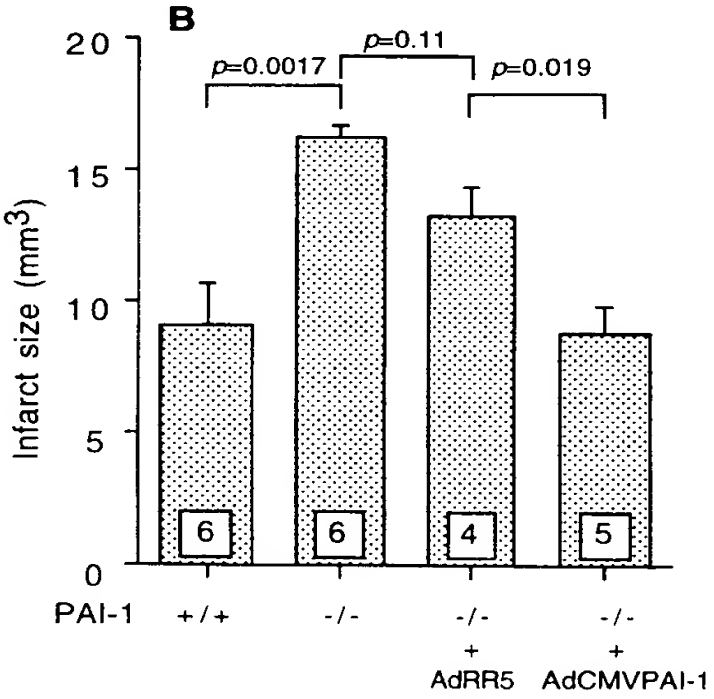
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Fig. 2B

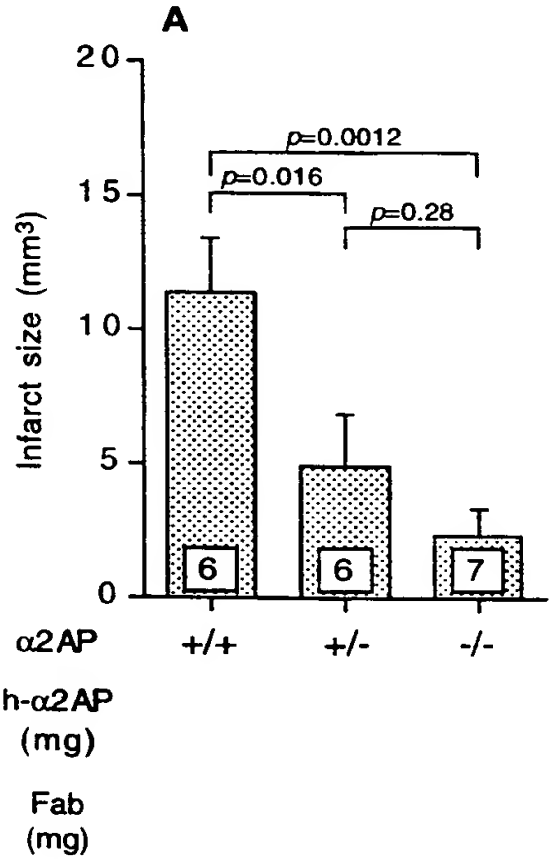
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Fig. 3A

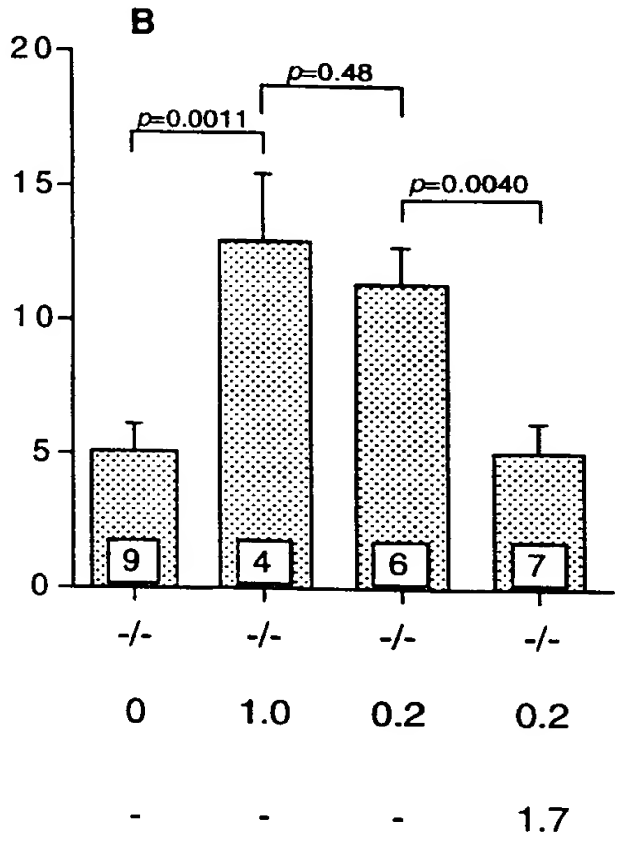
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Fig. 3B

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/EP 99/07405

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9812329 A	26-03-1998	AU 4413497 A	14-04-1998
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		EP 0937146 A	25-08-1999
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INTERNATIONAL SEARCH REPORT

tional Application No
PCT/EP 99/07405

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K39/395 A61K38/48 A61P9/10

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 98 12329 A (G. REED) 26 March 1998 (1998-03-26) page 23, line 25 -page 24, line 9 examples 1,2 claims ---	1-6
X	EP 0 631 786 A (IMMUNO AKTIENGESELLSCHAFT) 4 January 1995 (1995-01-04) claims --- -/--	6

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
23 February 2000	01/03/2000

Name and mailing address of the ISA	Authorized officer
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	A. BUTTE ET AL.: "Alpha2-antiplasmin causes thrombi to resist fibrinolysis induced by tissue plasminogen activator in experimental pulmonary embolism." CIRCULATION, vol. 95, no. 7, 1 April 1997 (1997-04-01), pages 1886-1891, XP002062707 New York, NY, USA the whole document	1-5
A	G. REED: "Functional characterization of monoclonal antibody inhibitors of alpha2-antiplasmin that accelerate fibrinolysis in different animal plasmas." HYBRIDOMA, vol. 16, no. 3, June 1997 (1997-06), pages 281-286, XP002062706 New York, NY, USA abstract	1-5
P,X	N. NAGAI ET AL.: "Role of plasminogen system components in focal cerebral ischemic infarction: a gene targeting and gene transfer study in mice." CIRCULATION, vol. 99, no. 18, 11 May 1999 (1999-05-11), pages 2440-2444, XP002112003 New York, NY, USA the whole document	1-5

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF THE RECORDING OF A CHANGE

(PCT Rule 92bis.1 and Administrative Instructions, Section 422)

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NL-2517 GK The Hague
PAYS-BAS

Date of mailing (day/month/year)
03 April 2001 (03.04.01)

Applicant's or agent's file reference
L/VZ70/cm/3

IMPORTANT NOTIFICATION

International application No.
PCT/EP99/07405

International filing date (day/month/year)
24 September 1999 (24.09.99)

1. The following indications appeared on record concerning:

[X] the applicant [X] the inventor [ ] the agent [ ] the common representative

Name and Address
NOBUO, Nagia
E-317, Handa-cho 3776
Hamamatsu, Shizuoka 431-3124
Japan

State of Nationality: JP
State of Residence: JP
Telephone No.:
Facsimile No.:
Teleprinter No.:

2. The International Bureau hereby notifies the applicant that the following change has been recorded concerning:

[ ] the person [X] the name [ ] the address [ ] the nationality [ ] the residence

Name and Address
NAGAI, Nobuo
E-317, Handa-cho 3776
Hamamatsu, Shizuoka 431-3124
Japan

State of Nationality: JP
State of Residence: JP
Telephone No.:
Facsimile No.:
Teleprinter No.:

3. Further observations, if necessary:

4. A copy of this notification has been sent to:

[X] the receiving Office
[ ] the International Searching Authority
[ ] the International Preliminary Examining Authority
[ ] the designated Offices concerned
[X] the elected Offices concerned
[ ] other:

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland
Facsimile No.: (41-22) 740 14 35

Authorized officer
C. Cupello
Telephone No.: (41-22) 338 83 38

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Assistant Commissioner for Patents
United States Patent and Trademark Office
Box PCT
Washington, D.C.20231
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 24 May 2000 (24.05.00)	
International application No. PCT/EP99/07405	**Applicant's or agent's file reference** L/VZ70/cm/3
International filing date (day/month/year) 24 September 1999 (24.09.99)	**Priority date** (day/month/year) 29 September 1998 (29.09.98)
Applicant NOBUO, Nagia et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

27 April 2000 (27.04.00)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer Claudio Borton
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 338.83.38